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USE OF UNICELLULAR ALGAE FOR EVALUATION OF POTENTIAL AQUATIC CONTAMINANTS

ANNUAL REPORT

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AMRL-TR-76-65

This report has been reviewed by the Information Office (OI) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

ANTHONY A. THOMAS, MD
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RJ-4	p,p'dioctyldephrylamine
RJ-5	Phenyl-α-napthylamine
Lubrication Additives	Unsymmetrical Dimethylhydrazine
Hydrazine	Symmetrical Dimethylhydrazine

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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Selenastrum capricornutum was used as a test alga to determine the toxic and/or biostimulating effect of lubrication additives, and hydrazines in aquatic environments. Standard batch and continuous culture procedures were used. The results show the relative and absolute toxicity under standard laboratory conditions of the compounds tested.

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Selenastrum capricornutum
Continuous Culture Algal Assays
Toxic Responses

SUMMARY

The National Environmental Policy Act in conjunction with an increased concern for environmental protection has created a need to evaluate the possible adverse effects of new chemical compounds prior to their widespread use. Because of the role algae play as primary producers in the food chain, they are an excellent choice for biological assessment. Both batch and continuous culture algal assays are available, and the Standard Batch Algal Assay appears to be a relatively easy, sensitive and inexpensive method for evaluating chemicals that may be discharged or accidentally enter the aquatic environment.

The research reported here is part of an extensive program to evaluate the toxic and/or biostimulatory effects of jet fuels, lubrication additives, and hydrazines in aquatic environments. This year's emphasis has been on the development of a bioassay protocol, photosynthetic methods of assessment, and chemical and algal assessment of hydrazine.

PREFACE

This is the First Annual Report of work performed under the Air Force Contract AF33615-76-C-5005. Work under this portion of the contract covers the period September 1, 1975 to May 31, 1976. The project is entitled "Use of Unicellular Algae for Evaluation of Potential Aquatic Contaminants." Research was conducted by the Water Resources Laboratory, School of Engineering, University of California, Irvine. The investigation was designed to expand the knowledge of possible toxic and biostimulatory responses of unicellular algae to pollutants and to aid Air Force personnel in assessing the environmental impact of various materials which may be released into the aquatic environment.

The authors gratefully acknowledge the assistance of Dr. Barbara Gittins and Mrs. Marcia Coryell in the overall conduct of the study.

INTRODUCTION

Increasing emphasis on environmental protection has made it necessary to determine the possible adverse effects of chemical compounds before they are put into use. The determination of the possible adverse environmental effects must include testing against a broad range of plants and animals.

Freshwater algae are critical organisms because of their role as primary producers in all aquatic food chains. Several algal species recommended for use in algal bioassays have been shown to be sensitive to small quantities of contaminants and the methodology for using these algae as a tool in determining growth effects has been well established.

OBJECTIVES

The overall objective of this investigation was to determine possible effects of selected chemicals which are currently being proposed for use and which may be released into the aquatic environment. The specific objectives were to determine the possible effects on algal growth of three types of compounds:

1. Jet fuels
2. Lube additives
3. Hydrazines

The compounds tested during the present year are listed under Methods and Procedures.

The effects have been determined under standard laboratory conditions[1] using batch cultures of the test alga Selenastrum capricornutum in the presence of bacteria.

METHODS AND PROCEDURES

Bioassay Protocol

Bioassays conducted to determine effects due to the presence of a chemical commonly use lethality as the criterion of toxicity. Results are usually expressed in terms of Lethal Concentration (LC) within a specified time interval with a stated percentage of test organisms killed. For example, a 72 hour LC_{50} would be that concentration of toxicant at which 50% of the test organisms are killed in 72 hours. This criterion is not valid for algal bioassays due to continuing cell division and the difficulty in determining whether an individual cell is dead or alive. The fourteenth edition of Standard Methods for the Examination of Water and Wastewater[8] states that for the batch algal assay of specific toxicants, the maximum specific growth rate (μ max) in control flasks should be compared with

μ max in the test flasks and the Effective Concentration (EC) and Safe Concentration (SC) calculated on the basis of the percent decrease in growth rate at the different toxicant concentrations. A 72 hour EC_{50} is defined as the concentration which produces a specific effect (reduction of growth rate) in 50% of the test organisms within the first 72 hours. Presumably the growth rate at the EC_{50} concentration is 50% of the growth rate in the controls. The SC is defined as the maximum concentration of a toxicant which has no observable effect upon the test organism over a given period of time.

Maximum growth rates are notoriously hard to determine for Selenastrum under batch conditions because:

1) The maximum growth rate occurs very soon after the initial lag phase when cell numbers are too low to be monitored with good statistical reliability,

2) The true maximum may persist only for a very short time (e.g. less than 24 hrs) and could be completely missed unless growth is monitored continuously or at short time intervals,

In addition to calculating the EC and SC on the basis of growth rate, these concentrations are determined for the maximum standing crop parameters of cell number, total cell volume and dry weight. Results are plotted showing the percent growht reduction (or increase) as a function of concentration. This type of data is presented in Figure 1 and shows the effect of varying concentrations of copper, lead and manganese on cell number.

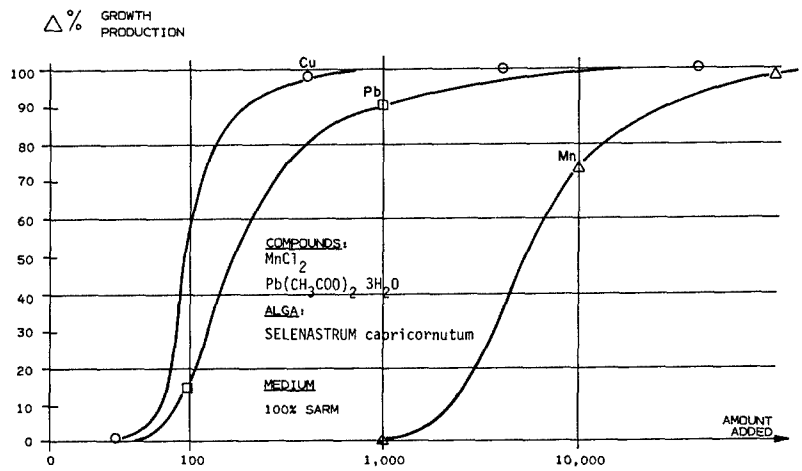

Figure 1. The Effect of Varying Concentrations of Metals on Cell Number of Selenastrum capricornutum

Batch algal bioassays were conducted in accordance with the "Algal Assay Procedure: Bottle Test (1971)"[1] as described previously.

Hydrazine Determinations

It is known that hydrazine and hydrazine derivatives break down in aqueous solutions so that a method for determining concentrations in the environment is of the utmost importance. For hydrazine itself, several methods are available but the spectrophotometric methods seemed the most sensitive and accurate.

The following conclusions have been reached after reviewing the Determination of Hydrazino-Hydrazide Groups by Hugh E. Malone[4] and various reprints received from the Air Force:

1. Gas chromatographic methods are used to separate mixtures of hydrazine but cannot be used for quantitative analysis in the 1 to 10 mg per liter concentration range because of lack of sensitivity,

2. The most sensitive quantitative methods for determining concentrations of hydrazine and methylated hydrazines involve colorimetric procedures.

Pesez and Petit[5] described the reaction between hydrazine and an acidified alcoholic solution of p-dimethylaminobenzaldehyde which results in the development of a characteristic yellow color. Watt and Chrisp[10] used this reaction as the basis for the quantitative method for the determination of hydrazine and a modification of this method has been used with good results. A method for the quantitative determination of UDMH is described by Pinkerton *et al.* in ASD Technical Report #61-708.[6] The method has a detection limit of 0.1mg per liter using 1cm cells. It is expected that this can be extended by a factor of 3 or 4 using 10cm cells.

Reagents and Apparatus

p-dimethylaminobenzaldehyde reagent (DMBA reagent)

100 mg absolute ethyl alcohol
4 g p-dimethylaminobenzaldehyde
10 ml concentrated HCl

Coleman Model 295 Spectrophotometer at 460nm

2 cm cells

Hydrazine stock standard (prepare daily):

dilute 1000µℓ hydrazine → 1 liter = 1000µℓ/ℓ

Hydrazine working standard:

dilute 100µℓ of stock standard → 100 mℓ = 1µℓ/ℓ

Procedure

A series of standards is prepared by adding 0.1 to 1.0 mℓ of hydrazine working standard to cuvettes. Sufficient deionized water is added to bring the total volume to 10 mℓ. Hydrazine concentrations will be in the range of 0.01 - 0.1μℓ ℓ. Deionized water is used as a blank. Add 1.0mℓ DMBA reagent to each tube and allow the color to develop for at least 20 minutes. Read the absorbance at 460nm. The color is stable for at least 3-4 hours.

Results

A plot of absorbance versus concentration (Figure 2) indicates that the method is linear to 0.08mg hydrazine per liter and that the detection limit is between .005-0.01mg/ℓ.

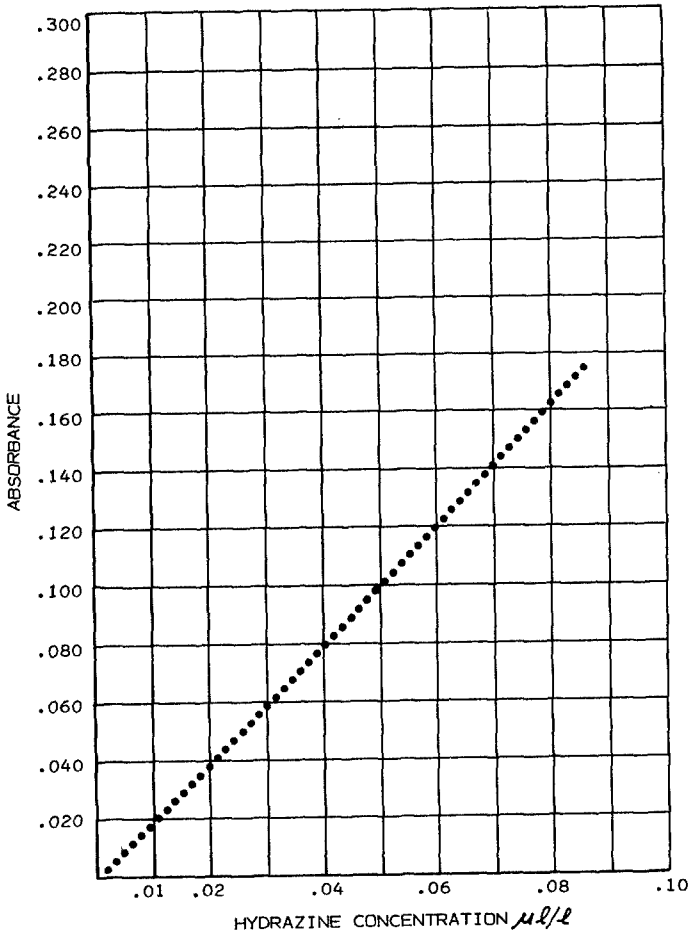

Figure 2. Method for Hydrazine Determination

This method can also be used for MMH but color development takes 1/2 hour and the color is stable for a shorter period of time.

GC/MS facility will be used to provide additional information about the compounds produced as hydrazine is degraded.

CONTINUOUS CULTURE

To establish a basis for future continuous culture bioassay evaluations, the kinetic constants of the Monod equation have been determined. The Monod equation is

$$\mu = \hat{\mu} \left[\frac{K_S}{K_s + S} \right]$$

where

$\hat{\mu}$ = maximum specific growth rate, and
K_s = half saturation constant.

This equation has been selected because it best describes the growth of the test organism Selenastrum capricornutum under continuous culture conditions. Because this equation is based on the assumption that one and only one nutrient limits the growth rate, different constants must be determined for each nutrient of interest. Phosphorus and nitrogen are the nutrients of prime interest at the present time.

For thirty days, six chemostats were operated in pairs at various residence time including 12, 15, 18, 21, 24 and 48 hours. They were operated on 100% SAAM which has a nitrogen to phosphorus ratio of 22.6 and therefore should be phosphorus growth rate limited so that $\hat{\mu}_P$ and K_P can be determined. Data including residence time (θ), pH, cumulative cell number, cumulative cell volume, orthophosphate and plate counts of bacteria were collected at regular intervals.

For 28 days, eight chemostats were operated at residence times of 15, 18, 21, 24 and 48 hours. They were operated under nitrogen limiting conditions using a modified SAAM with a nitrogen to phosphorus ratio of 5.0.

The kinetic constants of the Monod equation for the test organism Selenastrum capricornutum under phosphorus and nitrogen growth rate limiting conditions have been established. Subscripts "P" and "N" refer to phosphorus and nitrogen, respectively and the constants are:

$K_P = 0.0005 mg/\ell\text{-}P$
$K_N = 0.0173 mg/\ell\text{-}N$
$\hat{\mu}_P = 1.26\ day^{-1}$
$\hat{\mu} = 1.50\ day^{-1}$.

The development of these constants sets a basis for the use of continuous culture as a tool for the evaluation of potential aquatic contaminants provided that phosphorus or nitrogen is the growth rate limiting nutrient.

A critical assumption in the development of continuous culture theory is that the growth reactor is a Continuous Flow Stirred Tank Reactor (CFSTR). When a reactor is not acting as a CFSTR, then problems arise in determining the maximum growth rate ($\hat{\mu}$). This occurs because incomplete mixing will make the mean cell residence time (θ_c) less than the observed residence time (θ), and washout will occur at a lower residence time than under CFSTR conditions.

To determine if the growth reactors used in this laboratory are CFSTR, a tracer study was conducted. Using distilled water, two growth reactors were operated at a residence time of 1.0 day. Concentrated methylene blue was added to each reactor time t = 0 and initial concentration samples were taken. Samples were then collected every day for four days and the concentration of the dye remaining determined as a fraction of the initial concentration. Concentrations were determined on a Coleman Model 295 Spectrophotometer at a wavelength of 652nm.

A reactor which behaves as a CFSTR will have the tracer (methylene blue) removed according to the following exponential decay curve

$$\frac{C_t}{C_0} = e^{-\theta_t/\theta_o}$$

in which

C_t/C_0 = fraction of initial tracer remaining in reactor at time t

θ_t/θ_o = fraction of true residence period at time t

The theoretical decay curve and the tracer study data are shown in Figure 3. The results indicate that the growth reactors are CFSTR.

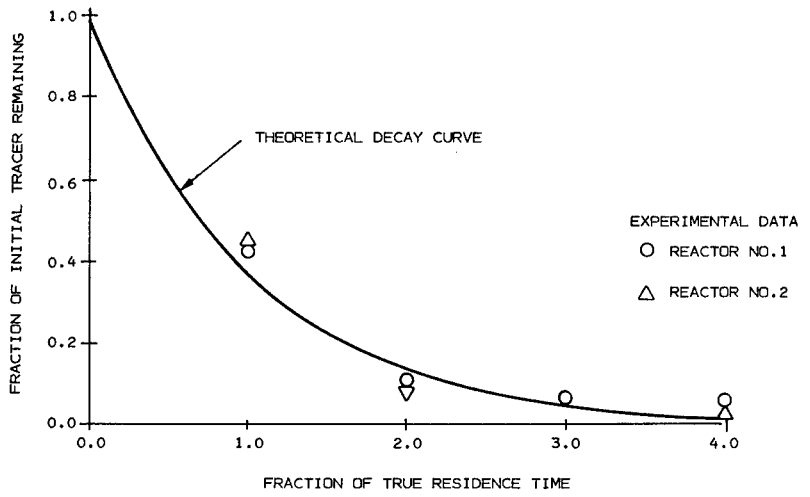

Figure 3, Comparison of Theoretical and Experimental Decay Curves

PHOTOSYNTHESIS/RESPIRATION

Both Warburg manometry and dissolved oxygen probe techniques were used for the studies of algal photosynethetic and respiration rates. Initially cultures of bacterized Selenastrum capricornutum were used, but when problems developed with replication, axenic cultures of Chlorella pyrenoidosa were substituted.

1. Warburg Experiments

The illuminated Warburg unit had to be modified in order to obtain proper temperature control for the studies of photosynthesis. Heat generated by the lamps was sufficient to cause significant temperature variations during the course of an experiment. Temperature control was accomplished by continuously circulating water from the illuminated Warburg unit through a refrigerated water bath.

The proposed experimental design for studies of algal photosynthetic rates, using Warburg manometry, was intended to test effects of different concentrations of 100% and 33% Standard Algal Assay Medium (SAAM) in combination with different concentrations of SDMH. Previous work had shown that a concentration of 1mg SDMH per liter had no effect on Selenastrum cell morphology while 10mg SDMH per liter initiated the morpholotical variants. Therefore a range of concentrations of SDMH between the 1mg and 10mg extremes were to be used. The results obtained from these experiments were to be compared with those of the algal batch assays.

Preliminary studies were conducted to determine optimum experimental conditions for normal algal cell metabolism during the Warburg experiments. It is known (1) that the metabolic rate differs when CO_2 is absent or present, (2) that in the presence of light both respiration and photosynthesis take place.

	Photosynthesis		Respiration
LIGHT =	$O_2 - CO_2$	+	$CO_2 - O_2$
DARK =	$CO_2 - O_2$		

In the presence of light, the observed rate of photosynthesis is less than the true rate because of oxygen uptake due to respiration. Removing carbon dioxide produced during respiration makes it unavailable for photosynthesis. Results obtained under these conditions cannot be compared with the natural situation where both gases are present. The rate of photosynthesis may be determined by the "steady state gas exchange" method described in Umbreit *et al.*[9] Briefly, this involves a period of equilibration followed by a period of dark (R1), a period of light (P&R), followed by another period of dark (R2). The true rate of photosynthesis is then calculated as $\mu\ell O_2$ produced per minute:

$$\text{Photosyn} = \frac{O_2 \text{ P\&R}}{\text{min. P\&R}} + \frac{1}{2}\left(\frac{O_2 R_1}{\text{min } R_1} - \frac{O_2 R_2}{\text{min } R_2}\right)$$

This procedure was used in an attempt to obtain information to meet the following objectives:

a. To determine whether the presence of the test compounds alters the rate of photosynthesis (P) or respiration plus photosynthesis (P+R) in axenic algal cultures over a given period of time.

b. To determine whether the abnormal Selenastrum cells photosynthesize and respire at the same rate as normal cells of the same age.

c. To determine whether abnormal and normal Selenastrum cells photosynthesize and respire at the same rate in the presence of varying concentrations of SDMH. Concentrations of 2, 4, and 8mg SDMH/liter of 100% and 33% SAAM were used.

Preliminary studies of respiration and photosynthesis using cultures of Chlorella pyrenoidosa indicated that cells had to be concentrated by centrifugation in order to provide a measurable response under the varying light-dark periods.

The first experiment designed to test the effects of hydrazine on photosynthesis was set up in the following manner: Experimental flasks containing 3.0mℓ of cell suspension and 0.5mℓ of buffer were established with 0.5mℓ of a 80μℓ/liter concentration of hydrazine in the sidearm, such that when the flask was tipped the final concentration would be 10μℓ of hydrazine per liter. Control flasks were established in the same manner, except that 0.5mℓ deionized water was placed in the sidearm.

The first respiratory period (40 minutes) and the first photosynthetic period (60 minutes) were run as determined in earlier tests. The hydrazine was then tipped into the experimental flasks and the deionized water into the controls. The next respiratory period and the following photosynthetic and respiratory periods all reflected the influence of hydrazine in association with the algal suspension. The results of this run are presented in Table 1.

TABLE 1

RESULTS OF FIRST WARBURG EXPERIMENT FOR DETERMINING THE EFFECTS OF HYDRAZINE ON PHOTOSYNTHESIS

FLASK	μℓ O_2 evolved per minute		% of control O_2 production	
	1st light period	2nd light period	1st light period	2nd light period
Controls (no hydrazine)				
Rep 1	1.187	1.950		
Rep 2	1.593	2.306		
$\bar{x}$	1.390	2.128	100	100
Experimental (hydrazine)				
Rep 1	1.511	0.427		
Rep 2	1.097	0.422		
Rep 3	0.337	0.042		
$\bar{x}$	0.982	0.297	71	14

The results show a marked depression in the photosynthetic rate as measured in μℓ O_2 produced per minute. Cells in the presence of hydrazine produced 29% less oxygen during the first photosynthetic period and 86% less during the second photosynthetic period, compared with the controls without hydrazine.

The next two runs were made with a slightly modified procedure. Rather than tipping the hydrazine into the experimental flasks before the second respiratory phase, the hydrazine was left in the sidearm until a respiratory period, a photosynthetic period, and a second respiratory period had all been established as the control period. In this way, all the flasks were both control and experimental. The second photosynthetic period and the last respiratory period thus were run in the presence of hydrazine and serve as the experimental period. The results obtained by this method are shown in Table 2.

TABLE 2

RESULTS OF MODIFIED WARBURG EXPERIMENT FOR DETERMINING THE EFFECTS OF HYDRAZINE ON PHOTOSYNTHESIS

Replicate #	$\mu\ell$ O_2 evolved per min.		% of control O_2 production
	controls (no hydrazine)	10$\mu\ell$ hydrazine/ℓ	
1	0.258	--	--
2	0.811	1.494	184
3	0.636	0.678	107
4	0.828	0.570	69
5	0.550	0.169	31
6	0.382	0.127	33
7	0.387	--	--
8	3.122	0.780	25
9	0.570	0.052	9
10	0.928	0.084	9
$\bar{x}$	0.848	0.495	58

The results show the same depression in the photosynthetic rate, with the exception of two flasks which showed an enhancement. The mean oxygen production was 42% less during the second photosynthetic period in the presence of hydrazine compared with the first photosynthetic period without hydrazine.

The Warburg investigations were later modified in order to improve experimental replication. These modifications included:

a) Using approximately twice the number of cells in the 3.0mℓ suspension placed in each flask.

b) The sequence of dark and light periods was continued to include two more light/dark

cycles which resulted in three normal periods of photosynthesis and one period of photosynthesis after the addition of the hydrazine.

c) The length of the light and dark periods was halved.

It had been expected that the denser cell suspension would result in more vigorous photosynthetic and respiratory responses and perhaps in better replication. However, this was not the case; the response was less than that obtained previously. The suspension may have been so dense that cells were shading each other and not getting enough light.

The extra dark and light period were added since previous investigations suggested that oxygen production was greater during the second light period. However, the length of the periods was not long enough to reach a steady state and this resulted in negative photosynthetic values.

After three months of using the Warburg technique to determine the effects of hydrazine on the photosynthetic rate of Chlorella pyrenoidosa, two facts have become evident:

a) Hydrazine does appear to have a depressing effect on photosynthesis when present at a concentration of 10μℓ per liter, since flasks containing hydrazine usually had a lower rate of oxygen production.

b) It is not possible to obtain credible quantitative results because of the questionable reliability of the Warburg apparatus, coupled with the variability in photosynthesis and respiration among the replicate flasks in a single run.

This variability may have resulted from the centrifugation and resuspension procedure which was necessary to concentrate the cells for measurable oxygen production in a reasonable period of time. It was subsequently decided that better quantitative results might be obtained using an oxygen electrode rather than the manometric technique.

Dissolved Oxygen Probe

Five available Princeton Aqua Science Aerobic Units were employed to measure dissolved oxygen production as a function of photosynthesis. Unfortunately, the accuracy of these electrodes was poor, the units would not remain calibrated, and the large volume of algal culture required for replicate runs made the method impractical. Although a few runs appeared hopeful, the overall data were questionable.

Later studies using International Biophysics Corporation BOD sensors were more promising. The results of a preliminary test run are as follows:

TIME	LIGHT/DARK	O_2 CONC. (in ppm)
9:00	L	3.50
10:00	L	4.15
11:00	L	4.95
12:00	L	5.20
1:30	D	4.60
2:30	D	3.10
2:50	L	4.65
3:00	L	4.80
4:00	L	6:30
5:30	L	8:00

Investigation showed that for practical short-term studies, cell concentrations in excess of 10^9 cells/liter had to be employed.

Electrodes which monitor dissolved oxygen and temperature were inserted into BOD bottles of algal suspension, placed between banks of fluorescent lights, after stripping dissolved oxygen from the medium by gentle bubbling with nitrogen. The subsequent response of the algae showed such a lag in oxygen production in the light and oxygen utilization in the dark that preliminary experiments extended over 24 hours, necessitating more multiple meters and automatic recorders than we were able to provide.

We utilized instruments available to us without major equipment expense but invested extensive effort and combined manhours without reaching the point where the effects of hydrazine on photosynthesis could be determined. Consequently, it was decided that the most efficient use of facilities and personnel required a return to batch techniques for dose response experiments with the various compounds using Selenastrum capricornutum as the assay organism.

COULTER COUNTER PROCEDURE

An aliquot of cell suspension is aseptically removed from the culture flask and diluted with a suitable electrolyte. Coulter product "Isoton" is the most satisfactory and convenient. The volumes of culture and electrolyte are dependent upon the cell concentration and aperture size; the aperture is dependent upon the particle (in this case cell) size. The standard conditions used for counting Selenastrum capricornutum are as follows:

Aperture size	70µm
Aperture current	1.6 milliamps (404KΩ)
Aperture current polarity	reversed each count
Manometer volume	0.5mℓ
Baseline channel	10 (2.52µm diameter)

The instrument is calibrated using known mono-sized particles such that some lower channel edge is set to a particular diameter. The upper and lower channel edges and mean channel volumes used are as follows:

Channel	Particle μm diameter range	mean volume μm^3
10	2.52 - 3.17	11.85
9	3.17 - 4.00	23.70
8	4.00 - 5.04	47.39
7	5.04 - 6.35	94.78
6	6.35 - 8.00	189.6
5	8.00 - 10.08	379.1
4	10.08 - 12.7	758.3
3	12.7 - 16.0	1516
2	16.0 - 20.2	3033
1	20.2 - 25.4	6066
0	25.4 - 32.0	12.13×10^3

Although the instrument presents cumulative (from channel 0 up to and including baseline channel) and differential population and volume data for each count, only the cumulative data have been presented in monthly reports. The reason is the time difference involved in recording two numbers per sample as opposed to 22 number per sample. Volume data readout on the Coulter Counter is expressed in $\mu m^3 \times 10^{-4}$. Mean cell size for the variant culture is:

$$\frac{(316 + 287) \times 10^4}{(121406 + 111907)} = 25.89\ \mu m^3/\text{cell}$$

Mean cell size for the normal Selenastrum culture is:

$$\frac{(552 + 541) \times 10^4}{(81826 + 80433)} = 67.36\ \mu m^3/\text{cell}$$

In order to present cell number and volume data per volume of culture, the data must be multiplied by the appropriate dilution factor. In this case, 0.5 mℓ of diluted sample was counted and 1 mℓ of sample was diluted with 20 mℓ of electrolyte. The dilution in this case was 42.

Compounds Tested

The following compounds, listed with source, lot number and anlytical information when known, were tested.

RJ-4 Sent from Wright Patterson AFB; transportation control number FY1455 4045 0002xxx.

RJ-5 Sent from Wright Patterson with above compound.

Phenyl-α-naphthylamine - Sent from Wright Patterson AFB

Octyl-phenyl-α-naphthylamine - Stauffer Chemical Company; OPAN HITEC 544 LOT NY710714. Obtained from Dr. Greenhouse's laboratory, UCI.

p,p'-dioctyldiphenylamine - Matheson Coleman & Bell

Practical grade (UDMH)
DX 1811
P 7943

Hydrazine 95 + % - Eastman

Lot A30
95%

sym Dimethylhydrazine dihydrochloride 97% SDMH

Aldrich Chemical D16, 180-2
Lot #072947
SDMH
Analysis by IR; elemental analysis

Methylhydrazine, 98%

Aldrich Chemical
Lot #081447
One mole certified wt. by infraredspectroscopy

RESULTS

HYDRAZINE DECOMPOSITION

Hydrazine is a strong reducing agent which decomposes rapidly in the presence of reducible compounds. Therefore, when evaluating the toxic and/or biostimulatory properties of hydrazine, the effects of the testing environment on the decomposition of hydrazine must also be determined.

An experiment was devised[3] to determine the effects of aeration and nutrient concentration on the decomposition of hydrazine. Nutrient concentrations of 0, 10, and 100% SAAM were run in triplicate, both with and without aeration. The results are presented in Table 3.

Aeration did not statistically affect the rate of hydrazine decomposition, but the concentration of the medium was significant. The higher the concentration of the medium the greater the rate of hydrazine decomposition. Therefore, when evaluating effects of hydrazine, the decomposition of hydrazine as a function of time and medium concentration must be taken into consideration.

HYDRAZINE IMPACT

To determine the effects of hydrazine on algal growth, a series of tests have and will be run. All runs will be accompanied with direct quantitative analysis of hydrazine concentration. Nutrient concentrations of 1.0% and 33% were used in past assessments, but bioassays in 1.0% medium proved to have poor precision and were highly dependent upon the history of the seed culture. For these reasons, 10%, 33% and 100% SAAM was used for these and will be used for future studies. The first screening test using 10% SAAM with hydrazine concentrations in the range of 0, 0.05, 0.5, 5.0, and 10.0µℓ/ℓ has been completed, with tests on 33% and 100% SAAM underway. Results are shown in Table 4 and Figure 4.

The analysis of growth shows that there is a statistically significant (>90%) difference in the maximum algal cell volumes for 10% SAAM at hydrazine levels of 10, 5, and 0.5µℓ/ℓ. For hydrazine at the level of 0.05, there is no statistical difference on day 14 but by day 21 the hydrazine treated flasks were slightly higher than the controls and the difference is significant (>90%). This presents a complex situation which will have to be clarified through improvement measurements of hydrazine content and decomposition.

OCTYL-PHENYL-α-NAPHTHYLAMINE (OPAN)

Batch algal bioassays were set up to determine the effect of octyl-phenyl-α-naphthylamine (OPAN) on algal growth. Growth effects of OPAN were determined in nutrient levels of 1, 33 and 100% Standard Algal Assay Medium (SAAM). Three

TABLE 3

COULTER COUNTER® Model T Worksheet

SAMPLE: Algal cultures 91-t21 and 88-t-21; Cell number data

ELECTROLYTE: Isoton — DISPERSANT: None

EQUIPMENT / SERIAL	Aper. Dia.	Ser. No.	CALIBRATION DATA	Part. Dia.	W	±IA	A
Model T	70	26224	Dow latex	3.49	81	1.6	156
ORGANIZATION: Water Resources Lab							
OPERATOR: M. Coryell — DATE: Feb. 1976							

$k = d \sqrt[3]{\frac{2^w}{A}}$

± I A	1.6
CALIB. A	233
APERTURE DIA.	70

$\frac{A_2}{A_1} = \left(\frac{d_2}{d_1}\right)^3 2^{(W_2 - W_1)}$

SAMPLE DATA

Volume Mean μ^3	Volume μ^3	Diameter Log_{10}	Diameter μ	Channel (W)	#1	#2	#3	#4
.00575	.004091	$\bar{9}$.29 760	.198					
.0115	.008181	$\bar{9}$.39 794	.250					
.0231	.01636	$\bar{9}$.49 828	.315					
.0462	.03272	$\bar{9}$.59 862	.397					
.0925	.06545	$\bar{9}$.69 897	.500					
.1851	.1309	$\bar{9}$.79 931	.630					
.3702	.2618	$\bar{9}$.89 966	.794					
.7405	.5236	0.00 000	1.00					
1.481	1.047	0.10 034	1.26					
2.962	2.094	0.20 069	1.59					
5.924	4.189	0.30 103	2.00					
11.85	8.378	0.40 137	2.52	10	19365	18369	935	778
23.70	16.76	0.50 172	3.17	9	63679	59134	1896	1669
47.39	32.51	0.60 206	4.00	8	35124	31668	28859	29196
94.78	67.02	0.70 240	5.04	7	2834	2320	45369	44160
189.6	134.0	0.80 275	6.35	6	238	283	4533	4363
379.1	268.1	0.90 309	8.00	5	100	81	205	231
758.3	536.2	1.00 343	10.08	4	32	33	21	26
1516.	1072.	1.10 378	12.7	3	21	16	3	9
3033.	2145.	1.20 412	16.0	2	13	3	3	1
6066.	4289.	1.30 446	20.2	1	1	0	2	0
12.13×10^3	8579.	1.40 481	25.4	0	0	0	0	0
24.27×10^3	17.16×10^3	1.50 515	32.0					
48.54×10^3	34.31×10^3	1.60 549	40.3					
97.18×10^3	68.63×10^3	1.70 584	50.8					
194.4×10^3	137.3×10^3	1.80 618	64.0					
388.7×10^3	274.5×10^3	1.90 652	80.6					
777.4×10^3	549.0×10^3	2.00 687	101.6					
1.555×10^6	1.098×10^6	2.10 721	128.					
3.109×10^6	2.196×10^6	2.20 755	161.					
6.219×10^6	4.392×10^6	2.30 790	203.					
12.44×10^6	8.784×10^6	2.40 824	256.					
24.88×10^6	17.57×10^6	2.50 858	322.					
49.75×10^6	35.14×10^6	2.60 893	406.					
99.50×10^6	70.27×10^6	2.70 927	512.					
199.0×10^6	140.6×10^6	2.80 961	645.					
398.0×10^6	281.1×10^6	2.90 996	812.					
796.0×10^6	562.2×10^6	3.01 030	1024.					

TABLE 4

COULTER COUNTER® Model T Worksheet

SAMPLE: Algal cultures 91-t21 and 88-t-21; Volume data

ELECTROLYTE: Isoton — DISPERSANT: None

EQUIPMENT	SERIAL	Aper. Dia.	Ser. No.	CALIBRATION DATA	Part. Dia.	W	±IA	A
Model T		70	26221	Dow latex	3.49	8	1.6	156
ORGANIZATION: Water Resources Lab								
OPERATOR: M.Coryell — DATE: Feb. 1976								

$$k = d\sqrt[3]{\frac{2^w}{A}}$$

± I A			1.6	$\frac{A_2}{A_1} = \left(\frac{d_2}{d_1}\right)^3 2^{(W_2 - W_1)}$
CALIB. A			233	
APERTURE DIA.			70	SAMPLE DATA

Volume Mean μ^3	Volume μ^3	Diameter Log_{10}	Diameter μ	Channel (W)	#1	#2	#3	#4
.00575	.004091	$\bar{9}$.29 760	.198					
.0115	.008181	$\bar{9}$.39 794	.250					
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.0462	.03272	$\bar{9}$.59 862	.397					
.0925	.06545	$\bar{9}$.69 897	.500					
.1851	.1309	$\bar{9}$.79 931	.630					
.3702	.2618	$\bar{9}$.89 966	.794					
.7405	.5236	0.00 000	1.00					
1.481	1.047	0.10 034	1.26					
2.962	2.094	0.20 069	1.59					
5.924	4.189	0.30 103	2.00					
11.85	8.378	0.40 137	2.52	10	18	17	0	0
23.70	16.76	0.50 172	3.17	9	124	115	3	3
47.39	32.51	0.60 206	4.00	8	137	123	112	114
94.78	67.02	0.70 240	5.04	7	22	18	354	345
189.6	134.0	0.80 275	6.35	6	3	4	70	68
379.1	268.1	0.90 309	8.00	5	3	2	6	7
758.3	536.2	1.00 343	10.08	4	2	2	1	1
1516.	1072.	1.10 378	12.7	3	2	2	0	1
3033.	2145.	1.20 412	16.0	2	3	0	0	0
6066.	4289.	1.30 446	20.2	1	0	0	0	0
12.13×10^3	8579.	1.40 481	25.4	0	0	0	0	0
24.27×10^3	17.16×10^3	1.50 515	32.0					
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97.18×10^3	68.63×10^3	1.70 584	50.8					
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388.7×10^3	274.5×10^3	1.90 652	80.6					
777.4×10^3	549.0×10^3	2.00 687	101.6					
1.555×10^6	1.098×10^6	2.10 721	128.					
3.109×10^6	2.196×10^6	2.20 755	161.					
6.219×10^6	4.392×10^6	2.30 790	203.					
12.44×10^6	8.784×10^6	2.40 824	256.					
24.88×10^6	17.57×10^6	2.50 858	322.					
49.75×10^6	35.14×10^6	2.60 893	406.					
99.50×10^6	70.27×10^6	2.70 927	512.					
199.0×10^6	140.6×10^6	2.80 961	645.					
398.0×10^6	281.1×10^6	2.90 996	812.					
796.0×10^6	562.2×10^6	3.01 030	1024.					

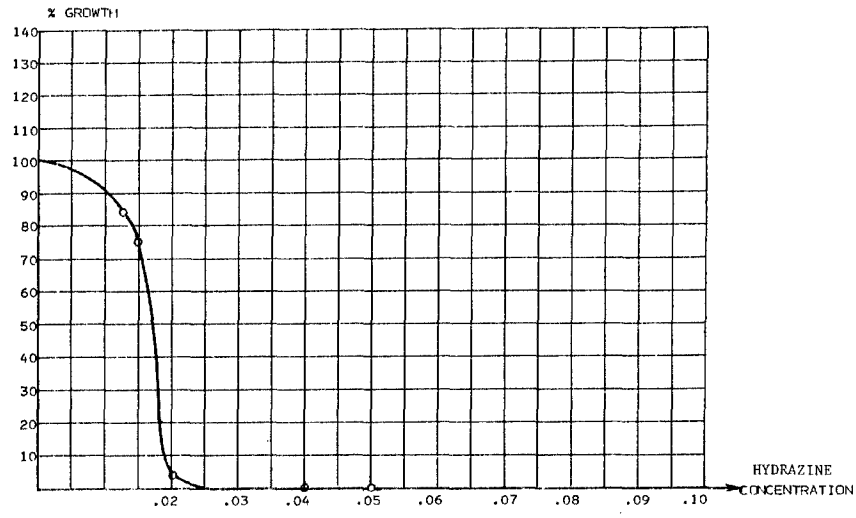

Figure 4. The Effect of Varying Hydrazine Concentration on Cell Number of Selenastrum capricornutum in 10% SARM

replicate flasks were set up with concentrations of 1, 10, 50, 100 and 500mg OPAN per liter at each nutrient level. Although these flasks were inadvertently seeded with a mixed culture of Selenastrum capricornutum and Chlorella sp, the data obtained provided information about effects on competition in different nutrient levels. Algal growth in the presence of the varying concentrations of OPAN was compared with growth in the control flasks without OPAN. These results will be compared with an identical batch experiment seeded with a pure culture of Selenastrum. It is expected that the results will include a minimum dose response but if OPAN does not have an effect on algal growth at these concentrations, the concentration will be increased until a significant response is evident.

MORPHOLOGICAL VARIANT

A morphological variant appeared when cells of Selenastrum capricornutum were exposed to SDMH, at a concentration of 10mg/ℓ.

Replicate cultures have been maintained of the variant and repeated miscroscopic examinations made of normal and varient cells of similar age. In addition, particle size-distribution analyses have been made using the electronic particle counter. Results of these studies show that, after more than 100 generations, cells derived from SDMH treated parent cells have retained the variant round-to-ovate shape without any sign of the arcuate horms seen in normal cells. The variant cells are 30% to 43% smaller than normal cells of similar age. Cultures of variant cells over a week old are readily distinguished from normal cultures of similar age by a difference in coloration.

An experiment was set up in an attempt to repeat the original SDMH experiment which gave rise to the morphological variant. The experimental design included seven replicate control flasks without SDMH and seven replicates each with concentrations of 5 and 10mg SDMH per liter of 33% SAAM. Data from the day of maximum growth are shown in Table 5. These results show that a concentration of 10mg SDMH per liter of 33% SAAM resulted in 83% fewer cells and 85% less total algal volume compared with the controls without SDMH.

Microscopic examination of algae from all seven flasks containing 10mg SDMH per liter revealed the abnormal spherical cells. A few normal cells were also observed in two of the

TABLE 5

MAXIMUM GROWTH DATA FROM SECOND SDMH BATCH ASSAY

Replicate Number	SDMH concentration (mg/ℓ)	Cell Number 10^6/ℓ	Total Volume mm^3/ℓ	Average cell size μm^3
1	10	571.8	21.6	37.8
2	10	666.2	26.5	39.7
3	10	67.5	3.36	49.8
4	10	454.2	12.7	28.0
5	10	351.4	18.1	51.4
6	10	190.1	7.98	42.0
7	10	75.4	2.52	33.4
$\bar{x}$ s.d		339.5 ± 238.1	13.3 ± 9.2	40.3 ± 8.4
1	5	1149	72.7	63.2
2	5	1182	69.3	58.6
3	5	1152	65.9	57.2
4	5	2082	130.2	62.5
5	5	1944	103.3	53.1
6	5	2005	119.3	59.4
7	5	1483	97.4	65.6
$\bar{x}$ s.d		1571 ± 428	94.0 ± 25.5	59.9 ± 4.2
1	0	1416	95.3	67.3
2	0	1314	87.8	66.8
3	0	1265	81.9	64.8
4	0	1926	114.7	59.5
5	0	1188	76.0	64.0
6	0	1326	91.6	69.1
7	0	1241	78.1	63.0
$\bar{x}$ s.d		1382 ± 250	89.3 ± 13.2	64.9 ± 3.2

flasks. The average volume of a normal cell was 64.9μm^3 compared with the variant which had an average size of 40.3μm^3.

A concentration of 5mg SDMH per liter did not cause the formation of large numbers of abnormal cells although a few cells were observed in one flask. A third large bioassay

was set up to determine with good statistical confidence whether variant cells developed from normal Selenastrum on exposure to SDMH or whether they were a result of contamination. Serial dilutions were made of the stock culture of Selenastrum and 100 replicate flasks containing 250mℓ of 33% SAAM were seeded with 9.56 cells per flask. If all seed cells were viable, it would have taken 14-15 doublings (divisions) to produce 1000 cells per mℓ and at this stage it was proposed that half of the flasks be treated with 10mg/ℓ of SDMH. Since it was considered unlikely that all cells were viable and that there is a lag phase of several hours during which newly seeded cells do not divide, it was concluded that it would take at least seven days before the flasks would reach 1000 cells per mℓ. This was based on one cell division/12 hours. However, by the seventh day some of the flasks were visibly green. Cell counts were made and the cell concentrations varied from 12,000 cells/mℓ to 81,000 cells/mℓ. Fifty flasks were randomly selected to receive 2.5mg of SDMH per 250mℓ while the other fifty served as controls and were left to grow normally. An additional 10 flasks received the 10mg/ℓ concentration of SDMH but were not seeded and in this way would serve as controls in the unlikely event that there were viable cells present in the SDMH. After 13 days of growth, cell numbers, mean cell size and total algal volume were determined for the 110 flasks as shown in Table 6.

TABLE 6

MEAN CELL NUMBERS AND VOLUMES IN 33% SAAM WITH AND WITHOUT SDMH

	No.Reps.	$\bar{x}$ Cell Number 10^6 cells/ℓ	$\bar{x}$ Algal Volume $mm^3/ℓ$	$\bar{x}$ Cell Size μm^3/cell
33% SAAM	48	2491	91.34	39.12
σ		370	13.26	
33% SAAM + 10mg SDMH/ℓ	21	1471	62.44	50.95
σ		701	29.18	

Two of the 33% SAAM flasks did not grow presumably because there were no remaining viable cells. Twenty-nine flasks which received the SDMH did not grow and there was no growth in any of the 10 flasks which received SDMH but which were not seeded. Samples were taken from a number of experimental and control flasks and examined microscopically. No variant cells were observed in any flask. No differences were seen under the miscroscope between the controls and the cells which had been exposed to SDMH. Coulter counter data indicate that SDMH treated cells were slightly larger than those in the control flasks.

Visual observation of the hydrazine batches suggests that the hydrazine compounds change with age even in the original unopened container. The first two batches were conducted using SDMH which had been transferred from the original container to a small vial. There was not enough remaining in the vial for the third experiment so SDMH from an original container having the same lot number was used. The results are inconclusive as to whether or not the variant developed because of exposure to SDMH.

FUTURE WORK PLANS

(1) The immediate task will be to carry out a further experiment with hydrazine using relatively low concentrations to elucidate additional data on the relationship between growth and concentration. From this, it will be possible to determine the SC and EC_{50}.

(2) As soon as this work with hydrazine is complete, similar series of experiments will be performed with UDMH and MMH.

(3) Protocols will be established for all previously tested compounds and for compounds as they are received in future.

(4) For hydrazine, the rate of decomposition is influenced by various ions. It is essential that the relative importance of the components present in the Standard Algal Assay medium be determined as quickly as possible.

(5) Initiate work on the effects of hydrazine and hydrazine derivatives on algal growth in marine situations, using a range of salinities to simulate estuarine, coastal and open ocean conditions.

(6) Develop criteria for the use in future of a "clean room" so that particularly dangerous compounds can be handled with safety.

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